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Protein Epitope Mimetics As Anti-Infectives

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Abstract:

There is growing interest in the design of synthetic molecules that mimic the structures and functions of epitopes found on the surface of peptides and proteins. Epitope mimetics can provide valuable tools to probe complex biological processes, as well as interesting leads for drug and vaccine discovery. One application of epitope mimetics is reviewed here, focusing on mimetics of the cationic antimicrobial peptides that form part of the innate immune response to microbial and viral infection in many organisms. Mimetics of these naturally occurring peptides and proteins may be useful to explore mechanisms of antimicrobial and immunomodulatory action, and as a potential source of new antibiotics to address one of the most pressing current threats to human health.

Introduction

A "protein epitope mimetic" is a conformationally constrained synthetic molecule that mimics the three-dimensional (3D) structure of that part of a folded peptide or protein recognized by its cognate biological receptor. This definition is closely related to the traditional view of an *epitope*, or "*antigenic determinant*", as being the part of an antigen recognized by antibodies and T-cell receptors (TCRs). The perspective here, however, is broader and includes not just structures recognized by antibodies and TCRs but also epitopes involved in ligand-receptor, protein-protein and protein-nucleic acid interactions (PPIs and PNIs). Most important are the 3D structures of epitopes and their mimicry in "semi-rigid" molecules. Protein epitope mimetics are interesting structurally and stereochemically, but more importantly, as tools to interrogate biological systems and as leads in drug and vaccine discovery.

Various levels of epitope complexity can be considered, starting with epitopes formed by short polypeptide segments at the protein surface, including ß-turns, loops and stretches of helical- or ß-structure. ß-Hairpins include both a turn (or loop) and regions of regular ß-structure. Early successes in the design of ß-turn mimetics came in the 1980's and 1990's, whereas over the past decade several successful approaches to helical, ß-sheet and ß-hairpin mimetics have been described [1-5]. More complex is the mimicry of discontinuous epitopes, comprising multiple polypeptide chain segments lying adjacent on the protein surface and adopting multiple types of secondary structure; here remains an important challenge for the future [6], perhaps one that can be addressed through the evolving field of "foldamer chemistry" [7,8].

Protein epitope mimetics typically sit in a still relatively unexplored area of molecular space (ca. 0.5-5 kDa). Nature has evolved molecules in this size range, including many structurally complex polyketide and peptide-based natural products. On the other hand, an

alternative source of such molecules is found by taking the macromolecules and macromolecular assemblies of Nature as a source of inspiration for mimetic design. Furthermore, many synthetic protein epitope mimetics tend to be modular in structure. They can be constructed from building blocks, such as α- or ß-amino acids or peptoids, which can be linked together using robust and efficient methods. It then becomes straightforward, in principle, to exchange building blocks and so vary structures (and optimize properties) in a combinatorial fashion using parallel synthetic chemistry, something that is not so easy when starting from complex natural products.

Molecular recognition involving proteins is mediated by surface exposed secondary structure elements such as ß-turns, ß-strands, ß-hairpins and α-helices. Protein epitope mimetics, therefore, seem to be attractive starting points for the design of PPI (and PNI) inhibitors (Figure-1). Inhibitor design for such targets based on small drug-like molecules has often proven rather difficult [9]. Protein epitope mimetic design offers an alternative approach, which draws upon improving knowledge of the structures of PPI hot-spots and the mechanisms of PPIs [10]. Another area where protein epitope mimetics have great potential is in synthetic vaccine design. Here the epitope mimetic should indeed act as an *antigenic determinant*, not simply to mimic the structure and receptor binding properties of a specific target, but rather to stimulate the immune system to produce antibodies and/or cyotoxic T cells that recognize an invading pathogen.

In the remainder of this short review, the focus is on one application of protein epitope mimetics, for the discovery of novel anti-infective agents.

Antibacterial CAMPs

The WHO has identified antibiotic resistance as one of the greatest current threats to human health [11]. The reasons for this include the paucity of new antimicrobial drugs currently

under development and the rapid spread of drug resistant bacteria [12]. In 2009 the Infectious Diseases Society of America (ISDA) challenged the USA and EU to develop ten new licensed antibiotics within the next 10 years, called the 10x'20 initiative (www.idsociety.org/10x20.htm) [13,14]. In order to address this challenge, it is important to consider new approaches to antibiotics that complement and extend established methods, such as natural product discovery and modification, whole-cell screening of compound libraries, and *in vitro* screens developed using genomic, proteomic and structural data [15-17]. One alternative approach is the design of mimetics based upon the cationic peptide and protein effector molecules produced by cells in the innate immune system, which provide a first line of defense against microbial infection in birds, fishes, amphibians, reptiles, mammals (including humans), insects, and other organisms [18].

Cationic antimicrobial peptides (CAMPs), or so-called host-defense peptides, play important roles in the anti-infective defense mechanisms of many organisms, including those of animals that also possess a more complex adaptive immune system. They are typically short cationic peptides of ~10-50 residues, with a net positive charge of +2 to +10, and often share amphipathic properties (cationic/hydrophobic). They differ widely, however, in sequences and secondary structures (Figure-2) (for recent reviews see [19-21]). Nevertheless, their physical properties frequently allow CAMPs to bind and insert into phosholipid bilayers by "barrel-stave", "carpet" or "toroidal-pore" mechanisms, and so disrupt the membrane bilayer [19]. CAMPs typically possess broad-spectrum antimicrobial activity against Gram-negative and Gram-positive microorganisms, but in the micromolar rather than the nanomolar range often seen with clinically used antibiotics. Nature has evolved CAMPs with such properties most likely because they are are released directly at sites of infection by effector cells, and then act locally in a number of ways, including by selective perturbation of microbial cytoplasmic membranes. In contrast, the relatively high concentrations of CAMPs

needed for antimicrobial activity, their ready degradation by proteases in serum, and their higher toxicity, are major obstacles for their development and use in the systemic treatment of bacterial infections in humans.

The mechanisms of action of CAMPs, however, have additional layers of complexity. Firstly, the (outer) bacterial membranes are not simple bilayers containing phospholipids. The membranes of Gram-positive bacteria are typically covered in a thick layer of peptidoglycan, teichoic acid, and protein, whereas the outer membrane (OM) of Gram-negative bacteria are often asymmetric, with the outer leaflet comprising mostly lipopolysaccharide (LPS) [22]. Individual LPS molecules in the outer leaflet are then cemented together through coordination of the phosphate and carboxylate groups in lipid A to divalent cations such as Mg^{2+}. It seems certain that cationic molecules (such as peptides and aminoglycosides) would be attracted electrostatically to the OM, perhaps leading to a pre-concentration in the outer sheaf [23]. This pre-association with the bacterial surface has been implicated in the self-promoted uptake of peptides across the OM, perhaps through competion with Mg^{2+} for binding to LPS in Gram-negative bacteria like *E. coli* and *P. aeruginosa* [24]. But the molecular details of whether/how this might occur remain unclear. Certainly, many CAMPs act by disrupting microbial membranes, and in these cases both enantiomers tend to show the same antimicrobial activity, ostensibly due to a lack of chiral discrimination within the interior of the cell membrane. However, it is worth noting that some aminoglycosides remain active in both enantiomeric forms [25] although they target a chiral molecule - the ribosome [26]. Secondly, it is clear that in some cases CAMPs are able to penetrate the bacterial membrane(s), and gain access to protein or nucleic acid targets inside the cell, as reviewed elsewhere [27]. Thirdly, it should be remembered that many CAMPs also have broad functions in the immune systems of their hosts, in addition to their antimicrobial activity [20,21]. They act at the interface of the innate and adaptive immune responses, and

participate in multiple aspects of immunity, inflammation, wound repair, and in maintaining homeostasis [28,29]. Recently, it was shown that some natural host-defence peptides, as well as synthetic analogues, can trigger a range of immunomodulatory responses, including an ability to moderate Toll-like receptor (TLR)-mediated responses stimulated by pathogen-associated molecular patterns and to protect against lethal endotoxemia and infections in animal models [20,21,28,30,31]. Other host-defence peptides such as the defensins and LL-37 also have immunomodulatory activities that likely mediate protection in animal models. The detailed molecular mechanisms of these innate immune effects, however, remain poorly characterized.

These considerations suggest that CAMPs will be interesting starting points for peptidomimetic and foldamer design, with the aim of probing this biology and perhaps discovering molecules with interesting antimicrobial and/or immunomodulatory properties.

CAMP mimetics

Antimicrobial peptides

Many hundreds or thousands of linear synthetic peptides related to naturally occurring CAMPs have been reported that possess membrane lytic activity (e.g. [32-36]). However, it remains doubtful whether flexible linear peptides can be endowed with the properties, including high potency, target selectivity, low toxicity and stability in serum and in whole animals, required for drug development. On the other hand, these properties are easier to achieve with conformationally constrained peptidomimetic scaffolds. A lead can be taken from Nature, where many examples of backbone cyclic cationic peptide antimicrobial products are known [37]. For example, many analogues of natural products such as gramicidin S and tyrocidine have been reported, although their antimicrobial activities

(MICs) usually remain in the micromolar range, and like the natural products, many are also strongly hemolytic.

Antimicrobial peptidomimetics

Many synthetic peptidomimetic approaches to antimicrobial peptides have been described or reviewed recently [38-40], including those based on hairpin mimetics [41-44], ß-peptides [45-54], peptoids [55-59], oligomeric aryl amides and aryl ureas [60-63], as well as related oligomers [64], foldamers and polymers (Figure-3) [65-73].

Novel mechanisms of antimicrobial action

Of special interest are cases where mixed and/or novel mechanisms of antimicrobial action can be demonstrated for CAMP-derived peptidomimetics. Already many years ago it was noted that individual CAMPs differ widely in their ability to depolarize the cytoplasmic membrane potential in Gram-positive and Gram-negative bacteria [24,74,75]. Gramicidin S, for example, causes maximal depolarization around the MIC, suggesting that the lytic actions of this peptide are directly responsible for bacterial cell death. Other CAMPs, however, such as polymyxin, cause little membrane depolarization at concentrations around the MIC, suggesting that the mechanism of cell death may involve some event other than the breakdown of the membrane permeability barrier. More recently, the kinetics of bacterial cell killing by antimicrobial arylamide foldamers was shown not to correlate with the effects of these molecules on bacterial membrane depolarization, suggesting again a mixed mechanism of action [61].

An interesting family of mimetics modelled on the ß-hairpin structure of the CAMP protegrin I (PG-I) was shown recently to have a novel mechanism of action [44]. The mimetics, typified by L27-11 and POL7001, have a backbone cyclic structure constrained into a ß-hairpin conformation with a D-Pro-L-Pro template [1]. The discovery of L27-11 and POL7001 was enabled in particular by the availability of an efficient parallel synthesis

method, which allowed the screening of compound libraries based upon this scaffold, and stepwise optimization of antimicrobial activity. Although PG-1 is strongly membrane lytic [76], both L27-11 and POL7001 are non-lytic at micromolar concentrations, and yet have potent antimicrobial activity in the nanomolar range, but only against Gram-negative *Pseudomonas* sp. The activity is highly enantioselective, which strongly suggests that a (chiral) receptor is required for the antimicrobial activity [44]. A likely target was shown to be the ß-barrel OM protein LptD (also called OstA and Imp), which plays an essential role in the assembly of the LPS layer in the outer leaflet of the OM in many Gram-negative bacteria (Figure-4) [77,78]. The interaction of the peptidomimetics with *P. aeruginosa* LptD was proven by photoaffinity labelling [44], but the binding site has not yet been characterized in detail. LptD is known to form a complex with the lipoprotein LptE in the OM where together they are responsible for the transport of LPS from the periplasm into the outer leaflet of the OM [77,79,80]. No crystal structure of LptD (from any microorganism) is currently available. It is also unclear how LptD interacts with LptE and how LPS transport to the outer leaflet occurs. The results available so far support the hypothesis that the antimicrobial activity of the ß-hairpin mimetics is due to inhibition of the key transport function of LptD, which then blocks OM biogenesis. More detailed studies are now required to investigate the influence the mimetics have on LPS transport to the OM.

Other CAMPs that target OM proteins

Some naturally occurring antimicrobial peptides and proteins are known to interact with ß-barrel OM proteins in Gram-negative bacteria, in particular, the colicins and closely related bacteriocins and microcins [81]. Many of these peptides and proteins have been shown to hijack ß-barrel proteins, including porins (OmpA/C/F) and those used to transport vitamin B_{12} and iron across the OM, to gain access to the periplasm. Recently, an intrinsically unstructured region close to the N-terminus of bacteriocin ColE9a was observed in a crystal

structure inside the OmpF ß-barrel.[82] The killing mechanisms of these antibiotics, however, are unrelated to the mechanism of transport across the OM, and typically involve protein or nucleic acid targets in the cytoplasm [81]. On the other hand, no natural products (including peptides or proteins) have so far been reported that interact with LptD or related essential OM proteins, such as BamA/Omp85/YaeT [83]. This is all the more surprising given their essential functions in OM biogenesis, and their exposed position in the OM.

Peptidomimetics targeting CXCR4

Polyphemusin is a naturally occurring ß-hairpin CAMP (Figure-2) with antiviral activity against HIV-1 due to its ability to antagonize the chemokine receptor CXCR4; the major co-receptor used by T-cell-tropic (X4-) HIV-1 to invade T-lymphocytes. CXCR4 is a G-protein coupled receptor that binds a protein ligand called "stromal cell-derived factor-1" (SDF-1 or CXCL12). Polyphemusin has been used as a starting point for the discovery of potentially useful CXCR4 antagonists [84-88]. One example is a family of backbone cyclic ß-hairpin mimetics, which again exploit the D-Pro-L-Pro template and a disulfide cross-link to stabilize hairpin conformations. Recently, an X-ray crystal structure revealed how one hairpin peptide binds to an engineered form of CXCR4 (Figure-4) [89]. The ß-hairpin inserts deep into the SDF-1 binding pocket, where a network of polar, hydrogen-bonding and hydrophobic contacts between the ligand and the receptor are responsible for the specific high affinity interaction. The ligand-binding site on CXCR4 is formed by residues in the inward-facing, protruding walls of the seven transmembrane helical bundle, several extracellular loops, and the N-terminal segment. It will be interesting to discover whether other GPCRs, that also have protein ligands, possess binding sites with similar architecture. If so, the structures and properties of the ß-hairpin scaffold might be readily optimized for interacting with such sites.

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Figure-1. The starting points for protein epitope mimetic design are the products of structural and molecular biology (for example, *left*, the CD4-gp120-mAb crystal structure (PDB 2QAD); *right*, the human papillomavirus-like particle L1 capsid (PDB 1DZL)). Some potential areas of application of protein epitope mimetics are highlighted.

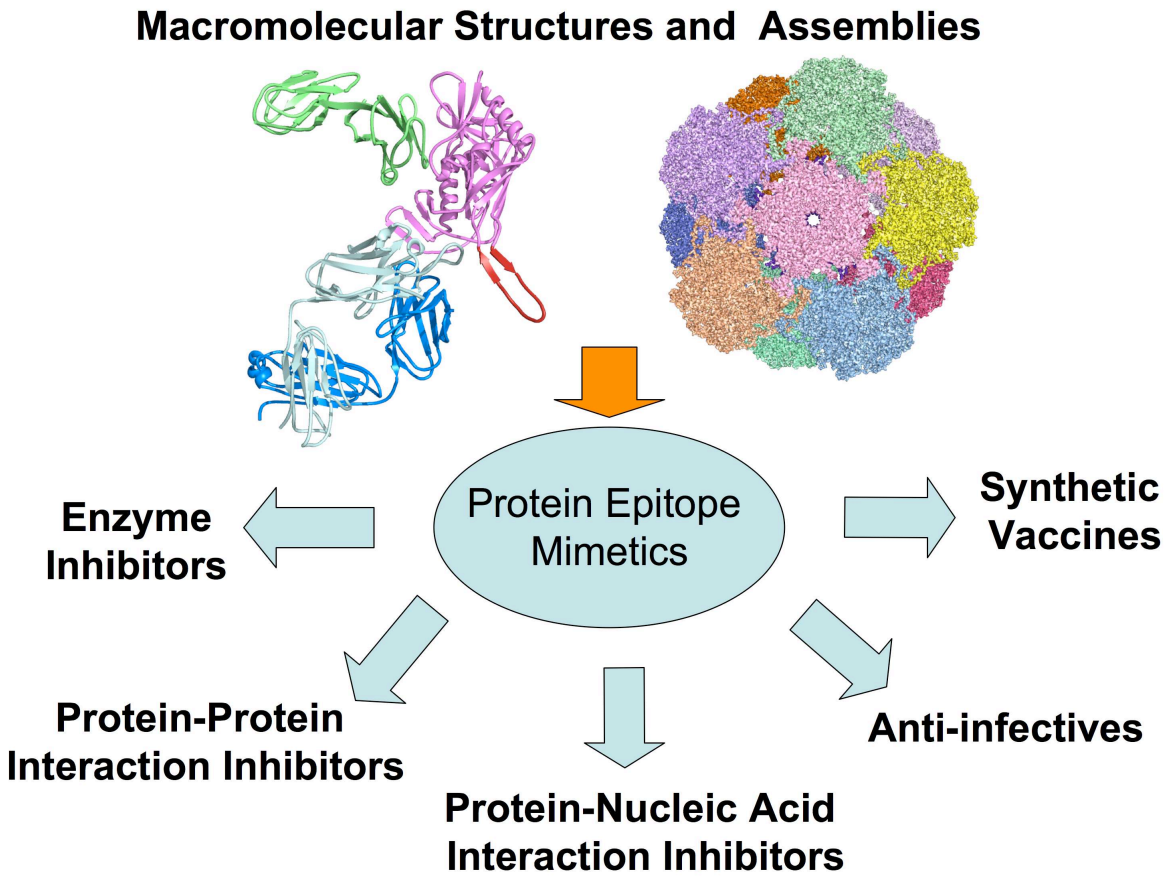

Figure-2. Cationic antimicrobial peptides come in a variety of shapes and sizes. A selection of structures is shown, with the name and PDB file.

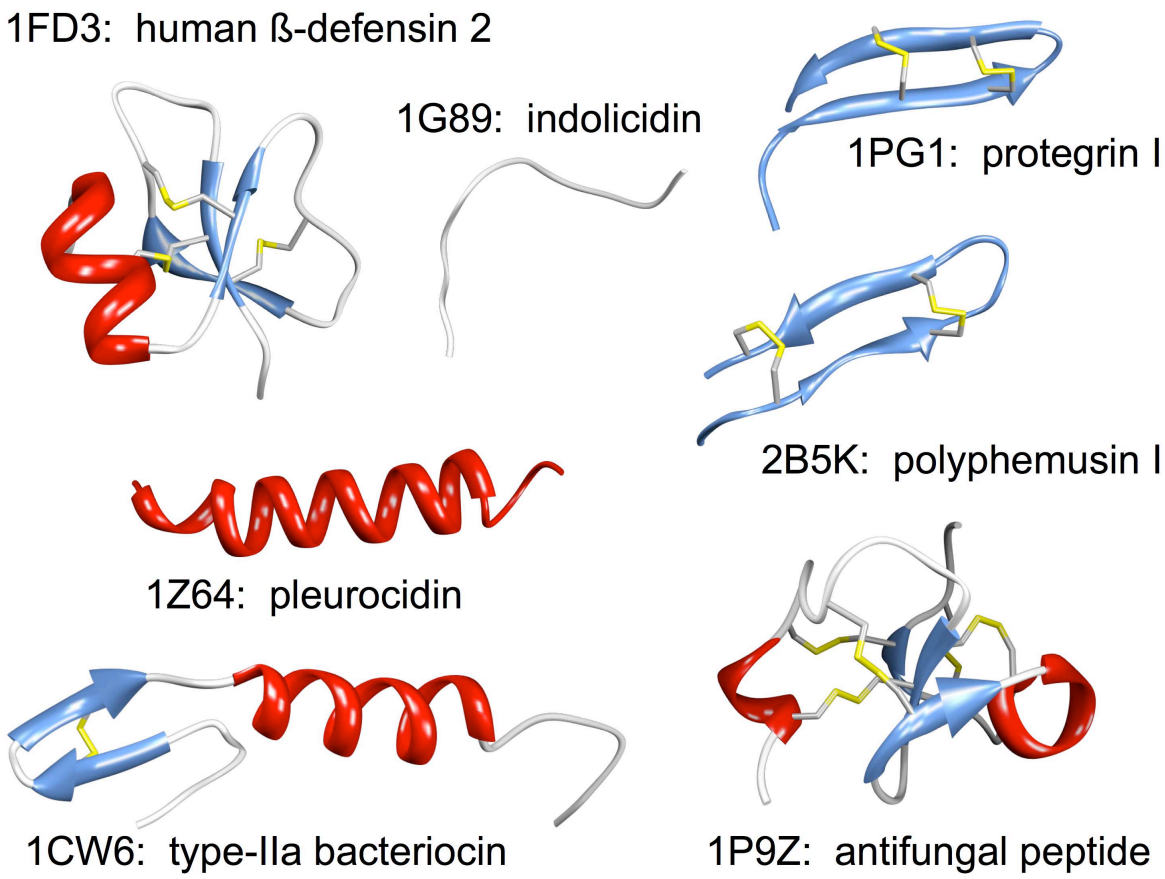

Figure-3. A selection of CAMP mimetics based on; **A**, oligomers and related; **B**, peptoids; **C**, ß-peptides.

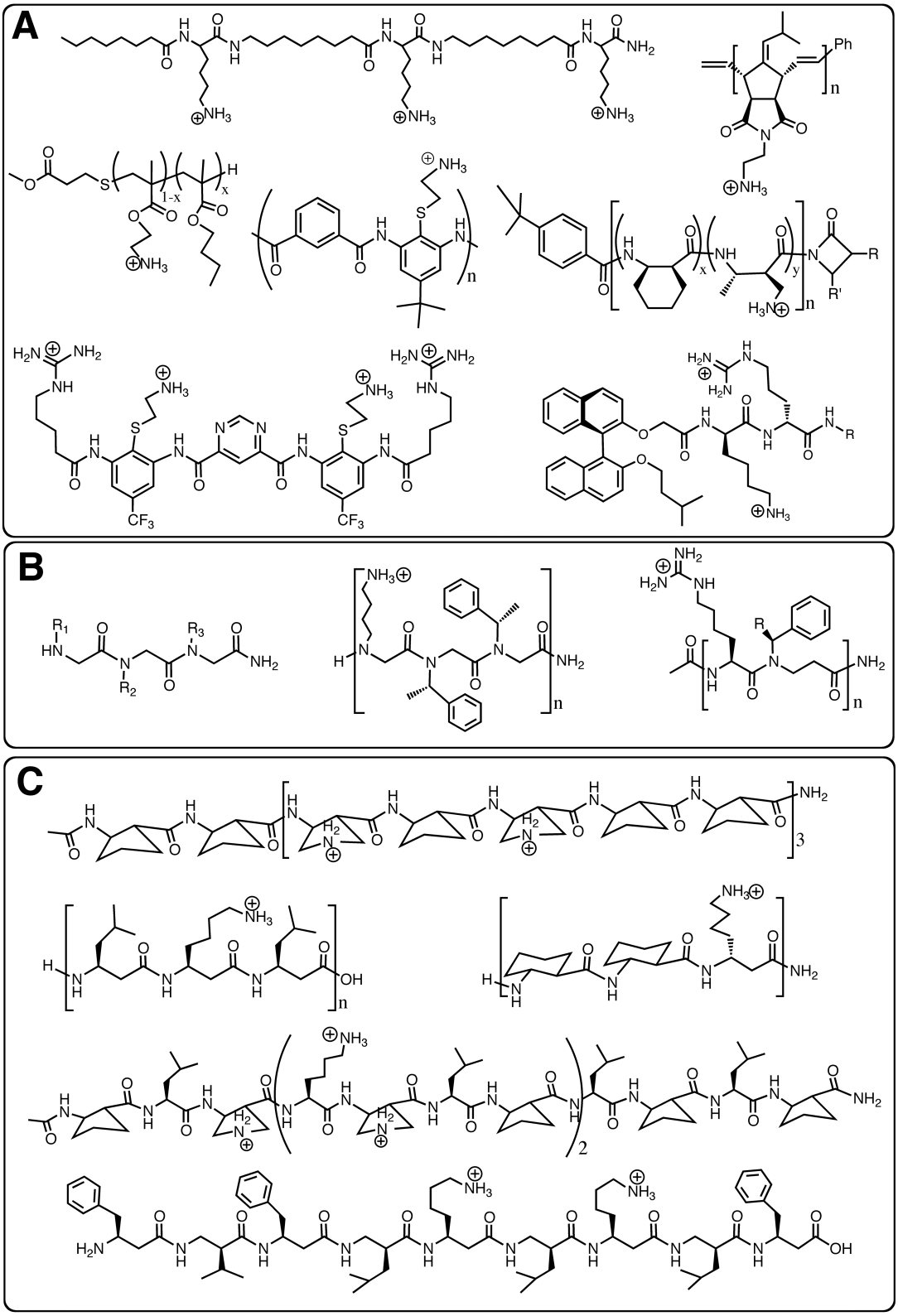

Figure-4. ß-Hairpin mimetics derived from protegrin I and polyphemusin (Figure-2) bind, respectively, to the bacterial outer membrane protein LptD [44] and the chemokine receptor CXCR4 [84]; *left*, LptD (red) is an OM protein, comprising a C-terminal ß-barrel (the ß-barrel shown is from PDB 2VQI) and an N-terminal periplasmic domain. LptD forms a complex with the lipoprotein LptE (green) [77,79,80]. The structure for LptE shown is that in PDB 2JXP (see text); *right*, the crystal structure of a ß-hairpin peptide (green with red surface) bound to the G-protein coupled receptor CXCR4 (PDB 3oe0) [89]. A slice through the complex is shown in a blue surface representation, with the full ribbon structure of CXCR4 in white. The image was made using the UCSF program *Chimera* [90].